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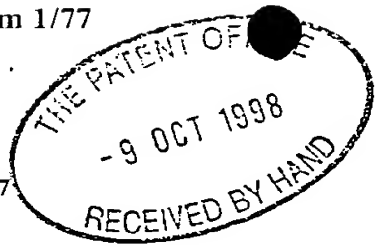

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120CT98 E396465-3 D02136
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1. Your reference SC/MH/N7444

2. Patent application number *(The Patent Office will fill this part)* **9822115.3**

3. Full name , address and postcode of the or of each applicant *(underline all surnames)*

KING'S COLLEGE
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The Strand
London WC2R 2LS

Patents ADP number *(if you know it)* 4001855001

If the applicant is a corporate body, give the country/state of its incorporation U.K.

4. Title of the invention

TREATMENT OF INFLAMMATORY DISEASE

5. Name of your agent *(if you have one)* Williams, Powell & Associates

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*
4 St. Paul's Churchyard
London
EC4M 8AY

Patents ADP number *(if you know it)* 5830310001 ✓

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Description	23
Claim(s)	1
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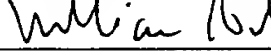
Signature  Date 08/10/98

12. Name and daytime telephone number of person to contact in the United Kingdom — Mr Lee Anderson 0171 329 4400

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TREATMENT OF INFLAMMATORY DISEASE

This invention relates to inflammatory disease and more particularly to rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovial joints which leads to joint destruction, disability and early death. Although the cause of RA is presently unknown, it has been suggested that type II collagen, uniquely found in the articular cartilage, is a possible autoantigen for RA. It has recently been proposed that gp39, a 39KD glycoprotein, and peptides derived from it , are such autoantigens. However, the data supporting this hypothesis are limited and the role of gp39 therefore remains uncertain.

The present invention stems from a different approach based on a study of chondrocytes, the specialised cells of articular cartilage. We have isolated a protein from human chondrocytes and human chondrosarcoma cell lines which reacts with antibodies present in RA patients' sera and meets the accepted criteria for a putative autoantigen. This purified protein has been tested for proliferation of T cells and has been shown to selectively proliferate synovial T cells from patients with RA. This protein is the immunoglobulin heavy chain binding protein BiP(78KD). Correct recognition of an RA autoantigen leads to the development of prognostic and diagnostic tests for this disease and specific therapy. We have isolated and sequenced the DNA for this protein. We have also cloned and expressed this DNA. The amino acid and DNA sequences are novel and are shown in Appendices given hereafter.

The first part of the following description concerns the characterisation of such an autoantigen; the second the cloning, sequencing and expression of the protein; and the third part the demonstration of disease (rheumatoid arthritis) and tissue (synovial compartment) T cell specificity to the autoantigen.

Part 1: CHARACTERISAtION OF AUTOANTIGEN

Chondrocytes/chondrosarcoma cells.

Chondrocytes were isolated from cartilage obtained during joint replacement. The cartilage was minced finely and digested with 1mg/ml collagenase. (Worthington). Following digestion the cells were centrifuged at 300g and resuspended in Dulbeccos minimal essential medium (DMEM)(Life Technologies, Paisley, UK) enriched with 10% foetal calf serum (FCS)(Harlan Sera-Lab, Loughborough, UK). Cell debris was washed off the adherent cells after 24hours and the cells allowed to expand until confluent. Cells were passaged using trypsin (0.25%) and split 1:3.

Chondrosarcoma cells (HTB94) (SW1353) were supplied by the American Type Culture Collection ATCC (Rockville, Maryland, USA) and by Dr J Block, Rush University, Chicago, USA (personal gift). These cells were cultured in DMEM with 10% FCS and split 1:3 after gentle trypsinisation (0.25% trypsin Life Technologies, Paisley, UK) when confluent.

Preparation of cell lysates.

Cells were scraped from the flask surface, homogenised and sonicated in the presence of proteinase inhibitors PMSF (2mM), leupeptin (200µg/ml) and aprotinin (50µg/ml) (Sigma, Poole, UK).

Sodium dodecyl sulphate (SDS) (Sigma, Poole, UK) was added to a final concentration of 1% and the proteins solubilised at room temperature for 1hour. Protein concentration was estimated by bicinchoninic acid assay using bovine serum albumin (BSA) as a standard protein (Sigma) and the cell lysate was used at 10µg/well equivalent.

Polyacrylamide gel electrophoresis (PAGE) and Western Blot.

The Mini Protean system (BioRad Laboratories, Hemel Hempstead, UK) was used to run the gels. 5, 7.5 or 10% SDS polyacrylamide denaturing gels 1.5mm thick were prepared (See Appendix 1). Gels were loaded with 10µg protein/well or the equivalent was loaded on preparative gels.

Electrophoresis was carried out at a constant 100V and broad range kaleidoscope markers (BioRad) were run in parallel with the cell lysates.

Following electrophoresis the proteins were blotted onto nitrocellulose at a constant 100V for 1hour (See Appendix 1). Nitrocellulose was then blocked with 3%BSA (Sigma, Poole,UK) and left at 4^0 C overnight. Preparative gel membranes were cut into 16 thin strips when necessary, each having an identical protein profile. The membranes were probed with patients sera (1/100 dilution) or specific monoclonal antibodies (at required concentrations) for 1hour at room temperature and then washed x3 over 1hour in TTBS (See Appendix 1). The secondary antibody, goat anti-human IgG (Fab^2) horse radish peroxidase(HRP) conjugate (Sigma) was added at 1/1000 dilution and incubated for 1hour at room temperature. The membranes were then washed x3 over 1hour in TTBS. Enhanced chemiluminescence (Amersham) was used to develop the system and antigen-antibody.HRP complexes appeared as discrete bands on photographic film when developed.

Isolation of the putative autoantigen p78

The band of interest was seen in approximately 30% of the rheumatoid arthritis sera used to screen the cell lysates as previously described.

To isolate the protein the cell lysate was concentrated x 23, using a 30,000MW cut-off filter(Vivascience). This protein was then loaded on 5% and 7.5% gels in parallel . One lane on each was loaded at normal concentration while the two other lanes were overloaded with the protein. Kaleidoscope markers were loaded on either side of the test lanes. The gels were then run as previously described until the kaleidoscope markers showed that the 70,000 MW protein would be in the bottom third of the gel as close as possible to the run-off point. The gels were then blotted onto PVDF membrane (Immobilon P, Millipore) which was immediately placed in distilled water after transfer of the proteins was complete. The strip with normal loading was used for immunodetection of the protein band. The developed film of this immunodetection and Ponceau red staining of the overloaded strips was used to identify the band on the membrane which was then air-dried.
These strips were then taken to isolate and sequence the protein using matrix assisted laser desorption ionisation (MALDI) spectroscopy .

The electroblotted proteins were stained with Ponceau S (0.05% w/v aqueous methanol/0.1% acetic acid) using a rapid-staining protocol (1). The dried, stained proteins were then digested in situ with trypsin (Boehringer, modified) and the peptides extracted with 1:1 v/v formic acid:ethanol (2). One 0.2 µl aliquot (approximately 5% of the total digest) was sampled and directly analysed by matrix-assisted laser desorption ionisation (MALDI) time-of-flight mass spectrometry using a LaserMat 2000 mass spectrometer (Thermo Bioanalysis,UK) (3). A second 0.2µl aliquot was quantitatively esterified using 1% v/v thionyl chloride in methanol and also analysed by MALDI to provide acidic residue composition (4). Native and esterified peptide masses were then screened against the MOWSE peptide mass fingerprint database (5). The remaining digested peptides (90% of total digest) were then reacted with N-succinimidyl-2(3-pyridyl) acetate (SPA) in order to enhance b-ion abundance and facilitate sequence analysis by tandem mass spectromety (6). Dried peptide fractions were treated with 7 µl 1% w/v N-succinimidyl-2(3-pyridyl) acetate in 0.5M HEPES (pH 7.8 with NaOH) containing 15% v/v acetonitrile for 20 min on ice. The reaction was terminated by 1 µl heptafluorobutyric acid (HFBA) and the solution immediately injected onto a capillary reverse-phase column (300 µm x 15cm) packed with POROS R2/H material (Perseptive Biosystems, MA) equilibrated with 2% v/v acetonitrile/0.05% v/v TFA running at 3 µl/min. The absorbed peptides were washed isocratically with 10% v/v acetonitrile/0.05% v/v TFA for 30 minutes at 3 µl/min to elute the excess reagent and HEPES buffer. The derivatised peptides were then eluted with a single step gradient to 75% v/v acetonitrile/0.1% v/v formic acid and collected in a single 4 µl fraction. The derivatised peptides were then sequenced by low-energy collison-activated dissociation (CAD) using a Finnigan MAT TSQ7000 fitted with a nanoelectrospray source (7,8). CAD was performed using 2.5 mTorr argon with collisonal offset voltages between -18V and -28V. The product-ion spectra were collected with Q3 scanned at 500 amu/sec.

RESULTS:

Sequence data obtained from 7.5% gel (single band)

from GR78_human
Specific identifying peptides:

NQLTSNPENTVFDAK 82-96
SDIDEIVLVGGSTR 353-366
TWNDPSVQQDIK 107-113

Identified human protein GR 78:
Kd glucose related protein precursor (GRP78)
Immunoglobulin heavy chain binding protein (BIP)

Part 2: CLONING, SEQUENCING AND EXPRESSION OF p78

1) mRNA isolation and PCR amplification of identified genes.

Human chondrocytes were isolated and cultured for three weeks as described. Poly(A) mRNA (1-2μg) was extracted with a Micro-Fastrack kit (Invitrogen) from a total of 1-2 X 10^6 cells. One microgram of the resulting mRNA was reverse transcribed into cDNA in a 20μl volume at 45°C for 1 hour using 1μl of Moloney murine leukemia virus reverse transcriptase (200u/μl); 5 X first strand buffer (Tris-HCl pH 8.3, 250mM; KCl 375 mM; MgCl 15mM); 0.1M DTT; oligo dT(12-18) 20ng/μl (Life Technologies); and dNTP mix 100mM (Amersham Pharmacia Biotec, Uppsala Sweden).

PCR was performed in a 50μl reaction volume under standard conditions (see below) using a Perkin Elmer Applied Biosystems thermal cycler PE2400. Primer sequences were derived from the GenBank database sequence corresponding to the Human gene for the immunoglobulin heavy chain binding protein, Bip (grp78), accession number X87949. Specific primers were synthesized to amplify the putative autoantigen gene from the chondrocyte cDNA. The resulting PCR product consisted of most of the grp78 coding region, bar the untranslated regions, signal sequences and the stop codon (nucleotide positions 279-2169 of the grp78 database sequence).

Primer sets for PCR were designed with integrated restriction sites to allow rapid subcloning of cDNA into the bacterial expression vector. The Forward primers encoded an NdeI site and the Reverse primers contained an XhoI restriction site:

Bip Forward primer 5'
TATACATATGGAGGAGGACAAGAAGGAGGACG 3' (32mer)
Bip Reverse primer 5'
CCACCTCGAGTTCTGCTGTATCCTCTTCACCA 3' (32mer)

After initial denaturation at 96°C for 2min the, PCR was performed for 28 cycles using a cycling profile of 94°C for 30s, 60°C for 30s and 72°C for 2min, with a final extension at 72°C for 7min. The PCR reaction generated a single specific Bip fragment of 1890bp.

2) Cloning of PCR generated fragments.

The restriction sites engineered into the forward and reverse primers used for the PCR reactions required flanking DNA for them to be recognised by their specific endonucleases (NdeI and XhoI). To provide this flanking DNA, the PCR generated fragment was cloned into a PCR cloning vector pCR2.1-TOPO (Invitrogen). The ligated plasmids were transformed into competent E.coli XL1-Blue (Stratagene) and plasmid DNA extracted using miniprep purification columns (Qiagen). The purified plasmid DNA for the clone was designated Bip-Topo. These DNA samples were stored at -20°C. The purified plasmid DNA for Bip-Topo was digested with NdeI and XhoI. The restricted fragments were separated by agarose gel electrophoresis and purified using the Qiagen DNA gel extraction kit.

3) Subcloning of restricted gene fragments into bacterial expression vector.

The purified fragment for the clone was ligated into the NdeI /XhoI pre-digested bacterial expression vector pET30a (Novagen). Ligation was performed at 12°C overnight in the presence of T4 ligase (20 units) and 1/10 vol of 10X ligase buffer (provided with the T4 ligase enzyme from Promega). The ligated plasmids were transformed into competent E.coli XL1-Blue (Stratagene) and screened by colony-PCR using Bip specific primers. Positive transformants carrying the required recombinant plasmids were purified and transformed into competent E.coli expression strain BL21-(DE3) (Invitrogen).

4) Sequencing of the 1890bp pET30::Bip subclone .

Sequencing of the 5' and 3' terminal ends of the pET30a::Bip clone confirmed that the recombinant DNA molecule was in-frame with the ATG start codon on the pET30 vector and that readthrough from this site continued through the Bip gene and ended with the 6X His residues and the stop codon located on the 3' arm of the expression vector.

Extensive DNA sequencing was performed using synthetic oligonucleotide primers spanning the entire length of the Bip subclone. Sequence analysis of the newly subcloned Bip gene fragment was performed by comparative alignment against the existing grp78 sequence from the database (accession number X87949). A number of differences between the two sequences were detected, both at the DNA and protein level (see Appendix 2). These areas of disagreement may either be a result of errors in the original DNA sequencing (of grp78) or they may indicate the presence of an additional related, but slightly different Bip gene in the genome.

All DNA sequencing was performed on an Applied Biosystems ABI 377 automated DNA sequencer using the dRhodamine dye terminator kit (Perkin Elmer- Applied Biosystems).

5) Expression of bacterial and purification recombinant proteins.

E.coli expression strain BL21-(DE3) containing the recombinant pET30a-Bip plasmid was grown at 37°C in LB medium containing kanamycin (50µg/ml). When the cells had reached an OD_{600} of 0.6units, isopropyl β-D-thiogalactopyranoside (IPTG) (1mM) was added to the medium to induce expression of the recombinant protein, driven by the IPTG-inducible promoter of the expression vector. To allow for maximal expression of the recombinant protein the culture was incubated for a further 4 hours at 37°C. The cells were pelleted by centrifugation and stored at -70°C.

For purification of the recombinant bacterial proteins the bacterial pellets were lysed in binding buffer (20mM $NaPO_4$, 500mM NaCl, 5mM Imidazole, 1mM PMSF, 1mg/ml Lysozyme, 5units/ml DNAse, 0.1% Triton X-100, pH7.4). The lysate was cleared by centrifugation to remove insoluble matter and cell debris. The cleared lysate was passed

over a binding buffer-equilibrated chelating Hi-trap affinity column, with a bed volume of 5ml (Pharmacia). The non-specifically bound protein was washed from the column under stringent conditions using a series of three wash buffers. The primary wash was performed using 100ml of Binding buffer. This was followed by a high stringency low pH wash (20mM $NaPO_4$, 500mM NaCl, 0.1% Triton X-100, pH5.5) and an additional high stringency wash using 100ml of 20mM $NaPO_4$, 500mM NaCl, 0.1% Triton X-100, 50mM Imidazole, pH7.4.
The histidine-tagged recombinant proteins were eluted from the column by stripping with 50mM EDTA. Eluted proteins were dialysed overnight against 1x PBS to remove EDTA and Ni contaminants. The purified protein was concentrated and washed in sterile PBS using a 50000 Mw cutoff concentrator column (Millipore). The total amount of protein was determined by spectrophotometry using BSA as a standard with the bicinchoninic acid assay (according to manufacturers instructions).

The concentrated Bip recombinant protein was resuspended in PBS to a final concentration of 10mg/ml and stored at -70°C in 50 µl aliquots.

Part 3: T CELL SPECIFICITY IN RA TO p78

Lymphocyte proliferation and gamma-interferon production by peripheral blood and synovial fluid mononuclear cells to the putative autoantigen p78

Mononuclear cells were separated from heparinised peripheral blood or synovial fluid by density centrifugation using Lymphoprep (Nycomed-Amersham, Little Chalfont, UK). Cells were washed x3 with Hanks buffered saline solution then used at $2x10^5$/well. Cells were cultured in RPMI 1640 supplemented with 10% heat inactivated human serum. Proliferation assays were carried out using control stimulants, tuberculin protein purified derivative (PPD)(Central Veterinary Laboratories, Weybridge, UK) at optimal concentration (10µg/ml) and pokeweed mitogen (PWM)(Sigma, Poole, UK)(2µg/ml) and a range of concentrations for recombinant human p78 BiP (1-50µg/ml). Cells were cultured for six days at 37^0 C in 5% CO_2. 100µl of supernatants for IFNγ estimation were withdrawn on day 5 and fresh medium used to replenish wells. Tritiated thymidine (^{3}HTdr) (0.2µCi/well) was added 24h prior to harvesting. Proliferation was determined by the uptake of

^{3}HTdr estimated on a Dry Matrix beta counter (Canberra Packard) and expressed as counts per minute. Interferon γ was measured by ELISA. Paired monoclonal antibodies to Interferon γ (IFN)(Pharmingen, Oxford, UK) were used for capture (1μg/ml) and detection (1μg/ml) of IFN γ. A standard curve was run on each plate using recombinant human IFNγ over a range from 0.01-10ngml^{-1}. Supernatants were used neat, 1/5, 1/10 and 1/100. The detection antibody was biotinylated, avidin-alkaline phosphatase conjugate was then linked to the biotin and developed with the substrate,3,3,5,5 tetramethylbenzidine (Sigma). The plates were read on a spectrophotometer at 450nm with a reference reading at 650nm.

1. T cell proliferation

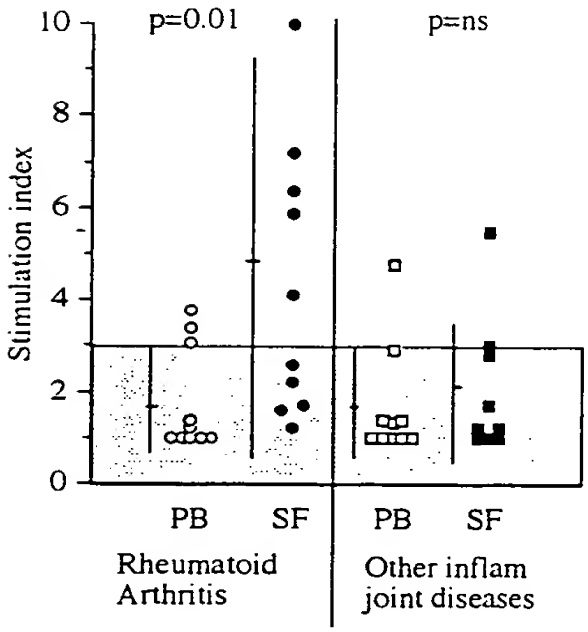
T cell proliferative responses were determined for mononuclear cell preparations from paired peripheral blood and synovial fluid samples. Ten samples from patients with rheumatoid arthritis and ten from disease controls were examined. In addition six HLA-DR4 positive control samples were incorporated in the study. Responses were determined to an optimum concentration of p78 and control antigens. The control antigens included purified protein derivative of Mycobacterium tuberculosis (PPD), pokeweed mitogen (PWM) and β-galactosidase (produced by the same method as p78 as a control for bacterial contamination).

Results show a significant increase in the synovial fluid responses to p78 in the RA patients compared to peripheral blood (Stimulation index 4.8±4.4 v 1.9±1.5: p=0.02 Mann-Whitney test). These data suggest a specific role for this protein in the synovial compartment of patients with RA.

Production of gamma-interferon (γ-IFN) was also determined for these samples and paralelled lymphocyte proliferation.

The DR4 positive control data show no difference in peripheral blood response when compared to disease control samples. Similarly no significant response was been to β-galactosidase in the RA synovial compartment, although responses were seen in control peripheral blood samples.

Proliferative response to p78



Proliferative responses to human p78 of mononuclear cells isolated from the peripheral blood and synovial fluid of patients with rheumatoid arthritis and other inflammatory joint diseases.

2. Use of tests for detection of antibodies to p78 in biological fluids or culture supernatants

Several techniques can be used, such as agglutination, Western blotting, and ELISA.

ELISA protocol for the detection of antibodies to p78 in sera

ELISA plates are coated half with p78 in bicarbonate buffer and half with buffer alone. The plates are blocked with bovine or human serum albumin (1% BSA or HAS) or other blocking protein before diluted sera are added to both the p78 coated half of the plate and also the non-coated half. After washing, biotin conjugated antibody to human immunoglobulin (polyclonal anti α, γ or μ) is added to the plate. Bound anti-immunoglobulin is detected with streptavidin-horse radish peroxidase and suitable substrate. Sera containing antibodies to p78 are determined spectrophotometrically. This test forms the basis of a diagnostic and/or prognostic test for rheumatoid arthritis.

3. Therapeutic application

There are several approaches to using p78 or derivatives for therapeutic purposes, including the following:

(a) Induction of mucosal tolerance.

Delivery of p78 autoantigen or peptides derived therefrom by mucosal routes, e.g. through the intestine or nasal mucosa, alters the immune response by downregulating disease activity leaving the patient's immune system otherwise intact. Alternatively p78 or p78 peptides can be delivered as DNA plasmids encoding them with an appropriate mammalian expression vector.

(b) Vaccination with TCR peptides

Peptides of the CDR3 region of the T cell receptor Valpha and Vbeta chains can be synthesised and used as vaccines for delivery by intradermal or intramuscular injection. Plasmids encoding these peptides can be used in the same way.

(c) MHC blockade with native or altered peptides

The p78 peptides may be given parenterally or orally in appropriate cases either unmodified or modified by amino acid substitution and/or attachment of chemical groupings so as to block MHC and especially HLA-DR4 thereby leading to suppression of T cell activation and disease. P78 peptides either native or altered may be combined with soluble HLA-DR4 molecules and applied parenterally or orally.

(d) Induction of tolerance by plasmid DNA immunisation

Plasmids consisting of DNA coding for whole p78 protein or its peptides linked to a mammalian expression vector may be given by injection. DNA coding for human IL-10, IL-4, IL-11, or TGF-beta, incorporated singly or in any combination, may be used to deviate the immune response to p78 towards a TH2 mode so as to suppress disease.

In the therapeutic regimes indicated above the protein or derived peptide may be administered in appropriate compositions delivering amounts ranging from about 0.1 micrograms to about 1 gram or the equivalent in the case of plasmid or vaccine preparations.

Appendix 1

Methodology for gel electrophoresis

Acrylamide gel: 10%

6.075ml acrylamide (40%) (BDH, Poole, UK)
3.35ml methylenbisacrylamide (2%)(Pharmacia Biotech, Uppsala, Sweden)
6.25ml acrylamide gel buffer (see below)
9ml distilled water (produced within the laboratory)
250µl Ammonium persulphate (AMPERS)(0.025mg in 250ul of distilled water)(Sigma-Aldrich, Poole, UK)
25µl NNN'N'-Tetramethylethylenediamine (TEMED)(Sigma-Aldrich, Poole, UK)

Acrylamide gel buffer: pH 8.8
1.5M Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)
pH titration with concentrated hydrochloric acid
0.4% Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

Stacking gel:
1.2ml acrylamide (40%)(BDH-Merck, Poole, UK)
0.65ml bisacrylamide (2%)(Pharmacia Biotech, Uppsala, Sweden)
3.15ml stacking gel buffer (see below)
7.5ml distilled water (produced within the laboratory)
125µl ammonium persulphate (AMPERS) (0.025mg/250µl)(Sigma-Aldrich, Poole, UK)
12.5µl NNN'N'-Tetramethylethylenediamine (TEMED)(Sigma-Aldrich, Poole, UK)

Stacking gel buffer: pH 6.8:
0.5M Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)
pH titration with concentrated hydrochloric acid
0.4% Sodium dodecyl sulphate (SDS)(BDH-Merck. Poole, UK)

Loading buffer:

2ml glycerol
2ml 10% Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)
0,.25mg bromophenol blue
2.5ml stacking gel buffer 4-times concentrated (0.5M Tris; 0.4% SDS; pH 6.8)
0.5ml 2-mercaptoethanol (Sigma-Aldrich, Poole, UK)

Electrophoresis/Running buffer:
3g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)
14.4g/l glycine (BDH-Merck, Poole, UK)
1g/l Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

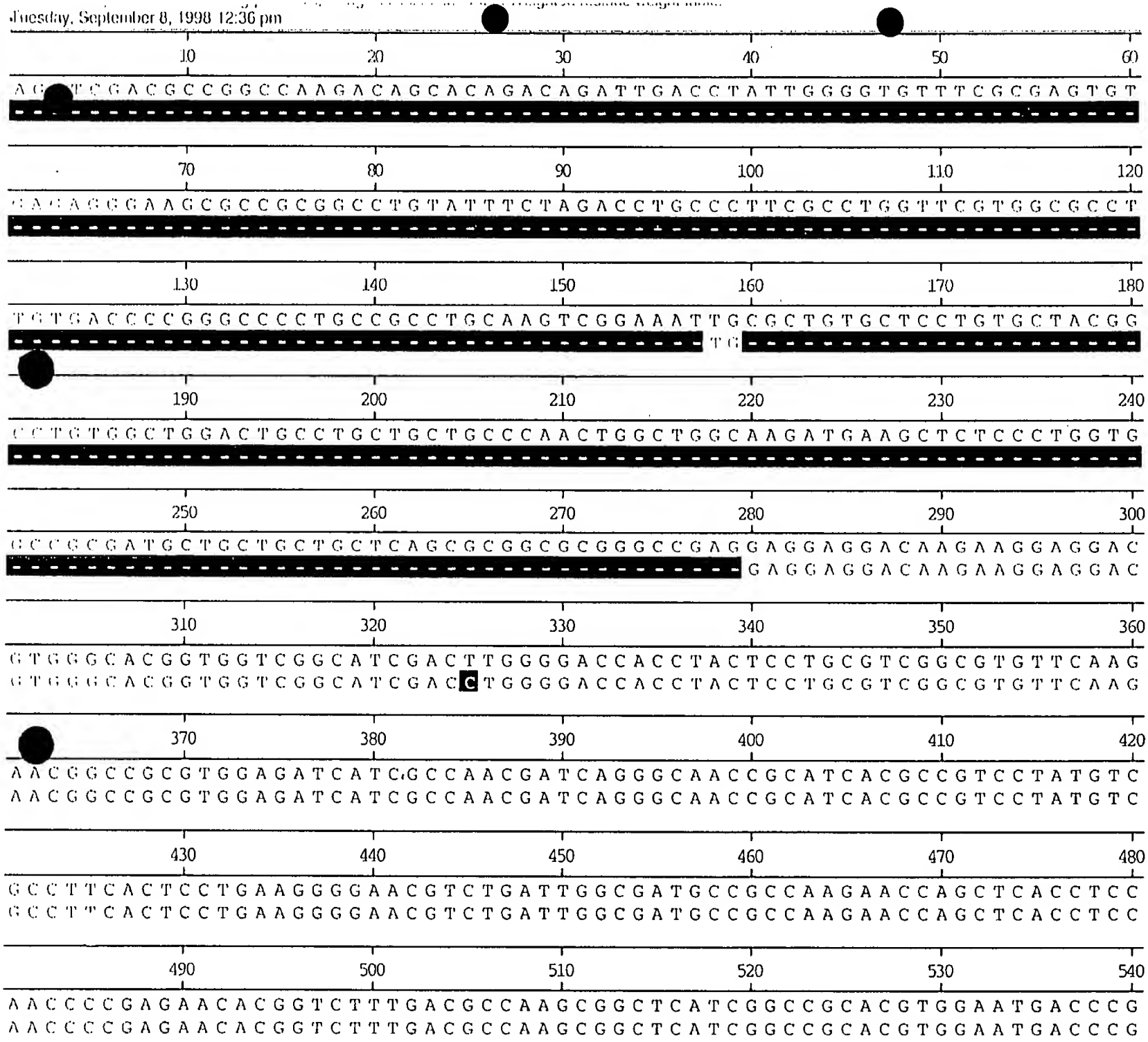
Transfer buffer:
3g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)
14.4g/l glycine (BDH-Merck, Poole, UK)
1g/l Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)
14.4g/l glycine (BDH-Merck, Poole, UK)
10% methanol (BDH-Merck, Poole, UK)

Tris Tween buffered saline (TTBS):
2.4g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)
29g/l sodium chloride (BDH-Merck, Poole, UK)
500µl tween 20 (polyoxyethylene-sorbitan mono-laurate)(Sigma-Aldrich, Poole, UK)

3% Bovine serum albumin solution (BSA):
3g Albumin bovine fraction 5 (BDH-Merck, Poole, UK)
100ml TTBS (see above)

Appendix 2

Cloned pET30:::Bip DNAa sequence aligned with grp78 DNA
(accession number X87949)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Database DNA seq X87949
Cloned pET30BIP seq
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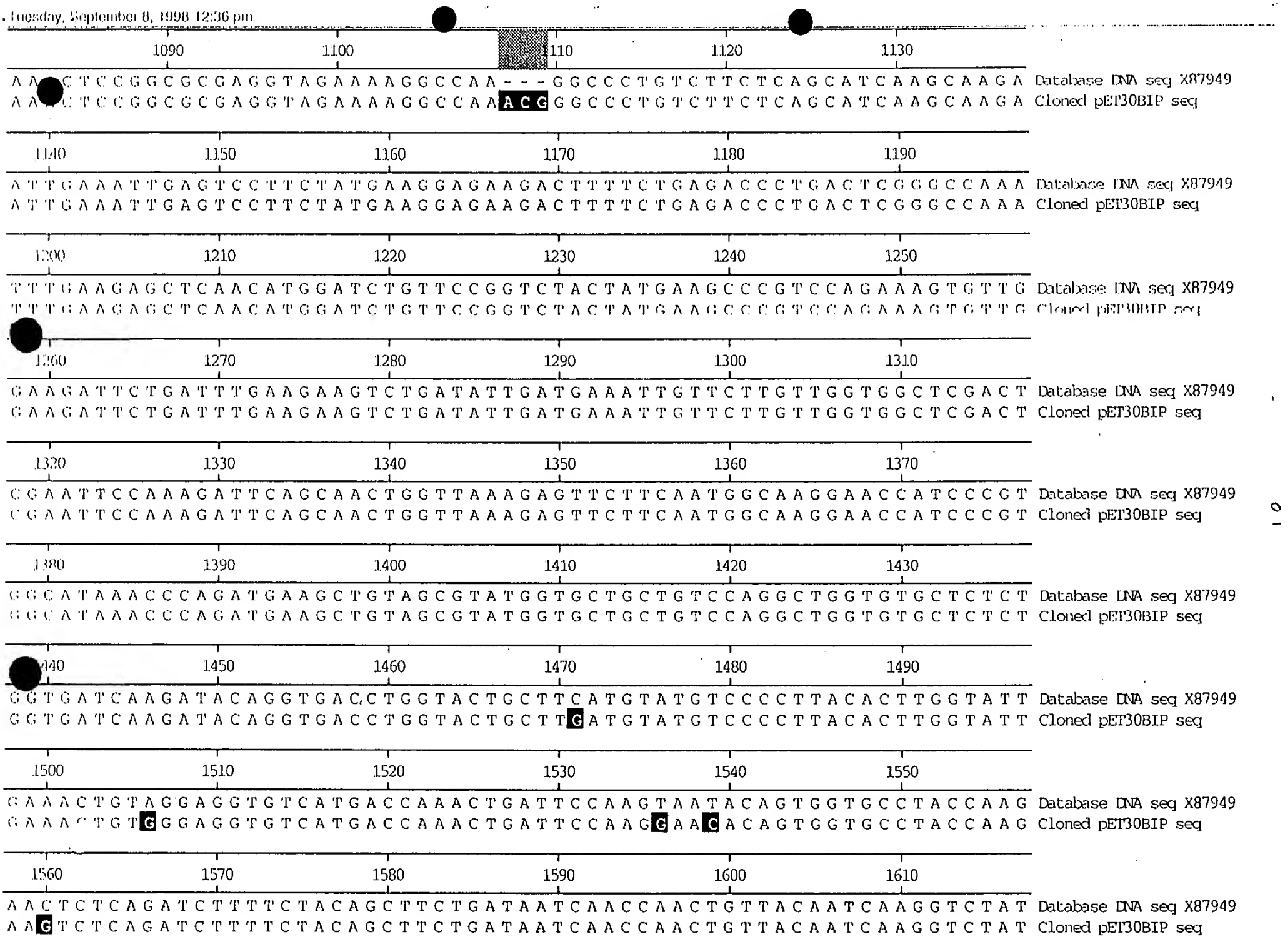
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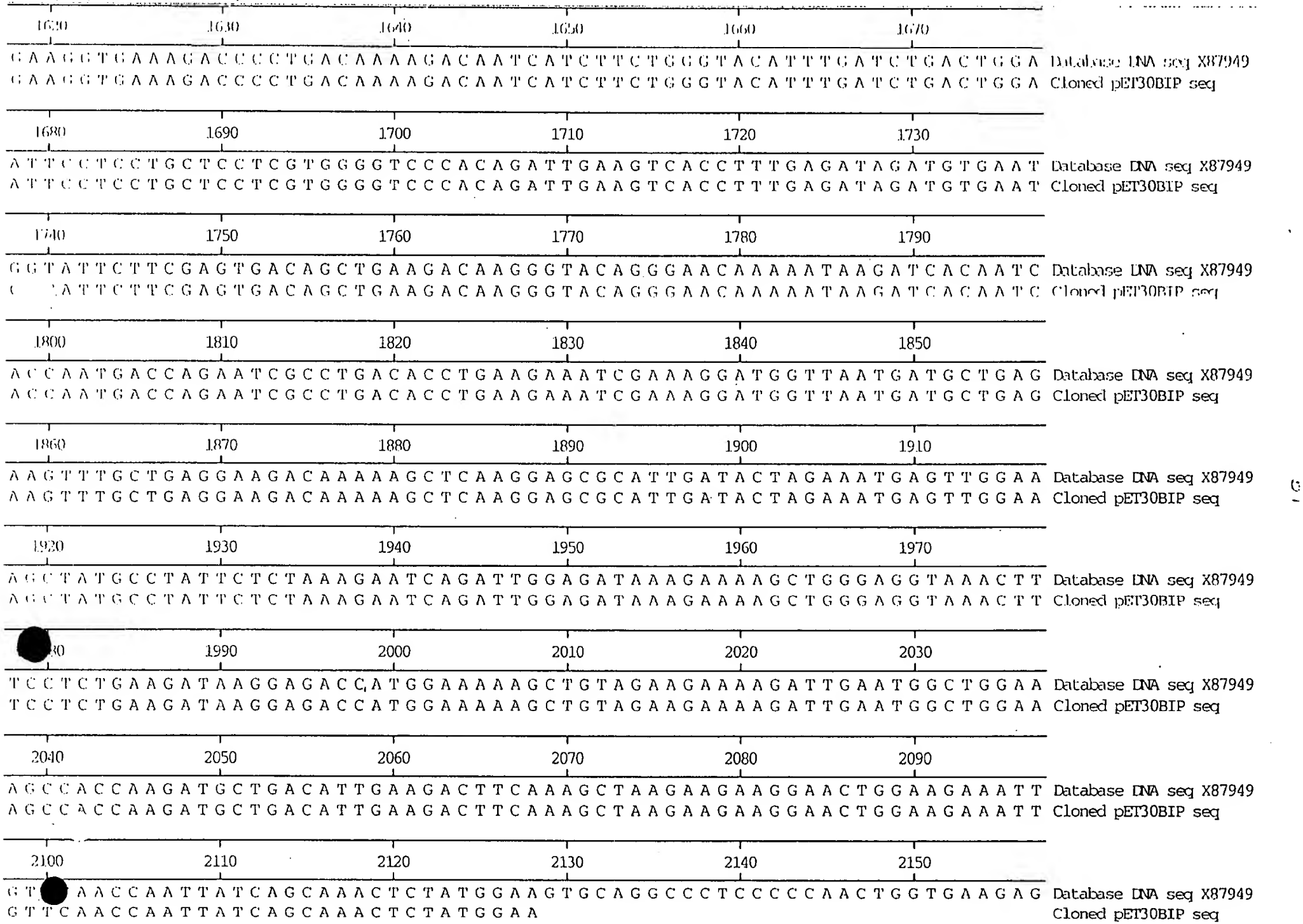
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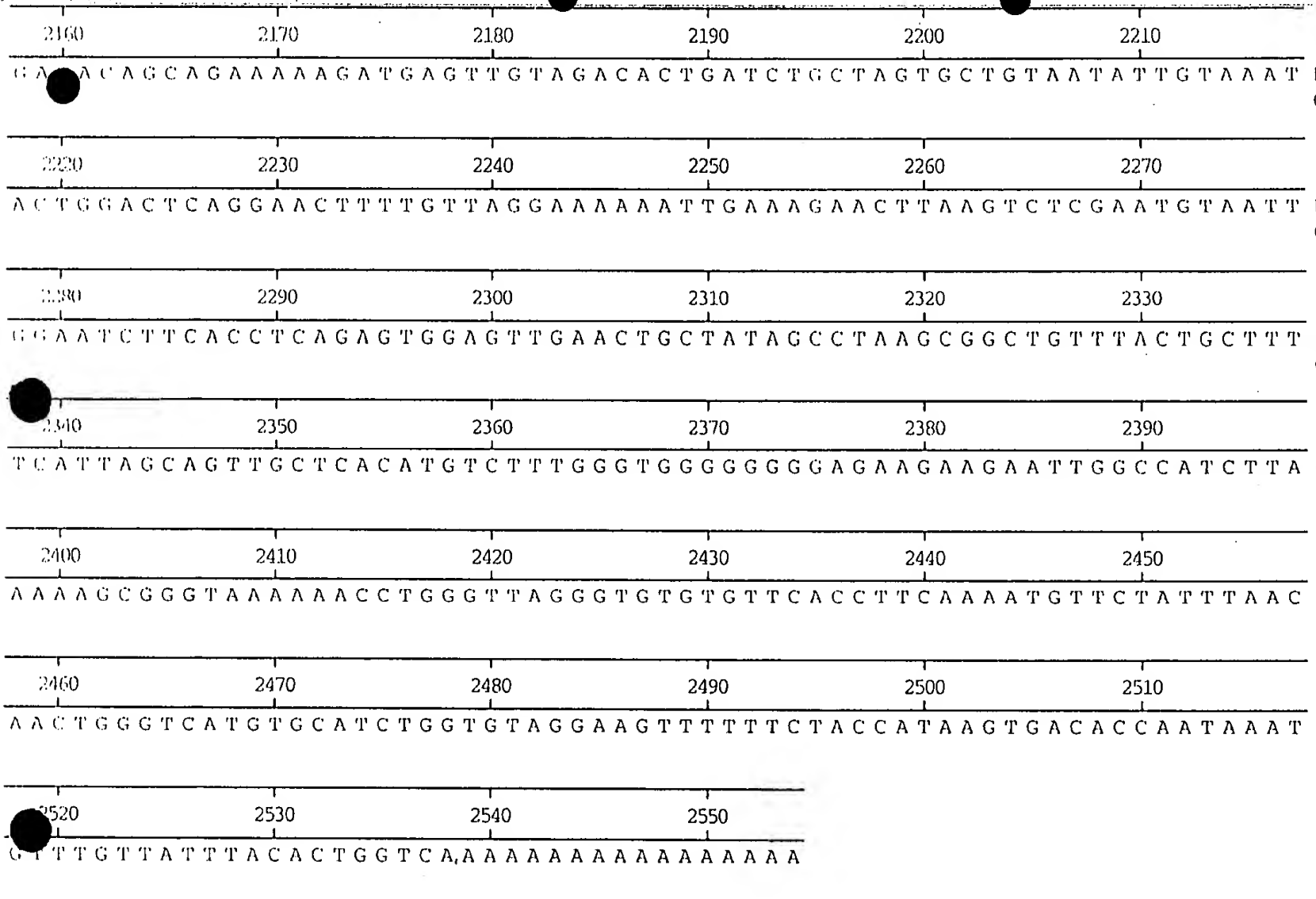
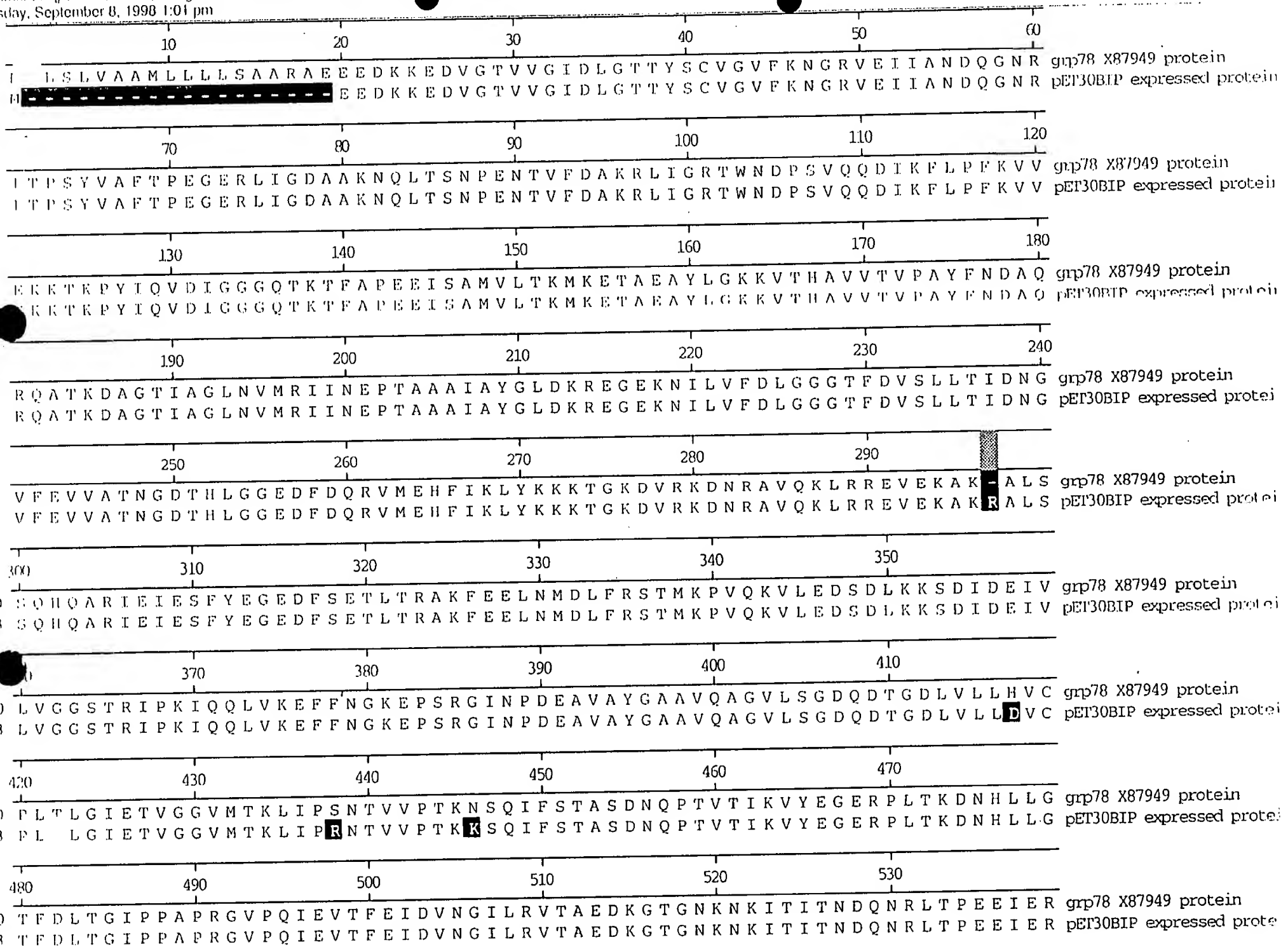


Decoration 'Decoration #1': Shade (with solid black) residues that differ from Database DNA seq X87949.

Appendix 3

Expressed pET30::Bip amino acid sequence aligned with predicted protein from grp78 DNA sequence (accession X87949)

Alignment Report of Untitled, using Clustal method with PAM250 residue weight table.
Tuesday, September 8, 1998 1:01 pm



Alignment Report of Untitled, using Clustal method with PAM250 residue weight table.
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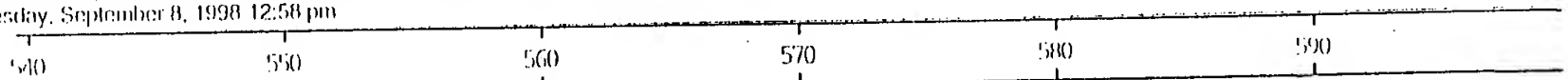

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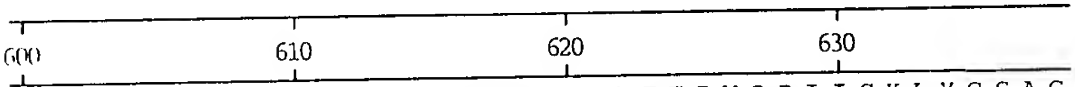

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Decoration 'Decoration #1': Shade (with solid black) residues that differ from grp78 X87949 protein.

CLAIMS

1. The use of immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom, as a marker for the presence of rheumatoid arthritis.

2. The use of protein BiP(78KD) according to claim 1, in which the protein has the amino-acid sequence as shown in Appendix 3.

3. The protein BiP(78KD) having the amino-acid sequence shown in Appendix 3.

4. A prognostic or diagnostic test for RA in which the test reagent for testing body fluid is immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom.

5. A test according to claim 4, being an ELISA assay.

6. Immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom, for use in therapy.

7. Immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom, for use in rheumatoid arthritis therapy.

8. A DNA sequence coding for Immunoglobulin heavy chain binding protein BiP (78KD), or for a peptide derived therefrom, for use in therapy.

9. A DNA sequence coding for Immunoglobulin heavy chain binding protein BiP (78KD), or for a peptide derived therefrom, for use in rheumatoid arthritis therapy.

10. A DNA sequence according to claim 9, containing the nucleotide sequence having the lower lines of sequence information shown in Appendix 2.

11. A recombinant vector containing a DNA sequence according to claim 9 or 10.